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REVIEW

The liver cancer immune microenvironment: Therapeutic implications for hepatocellular carcinoma

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Abstract

The liver is the sixth most common site of primary cancer in humans and the fourth leading cause of cancer-related death in the world. Hepatocellular carcinoma (HCC) accounts for 90% of liver cancers. HCC is a prevalent disease with a progression that is modulated by the immune system. Half of the patients with HCC receive systemic therapies, traditionally sorafenib or lenvatinib, as a first-line therapy. In the last few years, immune-checkpoint inhibitors (ICIs) have revolutionized cancer therapy and have gained an increased interest in the treatment of HCC. In 2020, the combination of atezolizumab (anti-programmed death-ligand 1) and bevacizumab (anti–vascular endothelial growth factor) improved overall survival over sorafenib, resulting in Food and Drug Administration (FDA) approval as a first-line treatment for patients with advanced HCC. Despite these major advances, a better molecular and cellular characterization of the tumor microenvironment is still needed because it has a crucial role in the development and progression of HCC. Inflamed (hot) and noninflamed (cold) HCC tumors and genomic signatures have been associated with response to ICIs. However, there are no additional biomarkers to guide clinical decision-making. Other immune-targeting strategies, such as adoptive T-cell transfer, vaccination, and virotherapy, are currently under development. This review provides an overview on the HCC immune microenvironment, different cellular players, current available immunotherapies, and potential immunotherapy modalities.

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; AFP, alpha-fetoprotein; Breg, B regulatory cell; CAF, cancer-associated fibroblast; CAR, chimeric antigen receptor; CCRK, cell cycle–related kinase; cDC, conventional DC; CIK, cytokine-induced killer cell; CLCF1, cardiotrophin-like cytokine factor 1; CLD, chronic liver damage; CTLA4, cytotoxic T-lymphocyte–associated protein 4; DC, dendritic cell; FDA, Food and Drug Administration; GPC3, Glypican-3; GZM, granzyme; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGF, hepatocyte growth factor; HSC, hepatic stellate cell; ICI, immune-checkpoint inhibitor; IFN, interferon; KC, Kupffer cell; LAG3, lymphocyte-activating 3; M-MDSC, monocytic MDSC; MDSC, myeloid-derived suppressor cell; NET, neutrophil extracellular trap; NK, natural killer; NKT, natural killer T; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; pDC, plasmacytoid DC; scRNAseq, single-cell RNA sequencing; TAM, tumor-associated macrophage; TAN, tumor-associated neutrophil; TCR, T cell receptor; TGFβ, transforming growth factor β; Th, T helper; TIB, tumor-infiltrating B cell; TKI, tyrosine kinase inhibitor; TLR, toll-like receptor; TME, tumor microenvironment; Tr1, type 1 Treg; Treg, regulatory T cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

INTRODUCTION

Liver cancer is the sixth most common cancer worldwide and the fourth leading cause of cancer-related death, with a 5-year survival of 18%.[1,2] Hepatocellular carcinoma (HCC) accounts for 90% of the cases.[3] Hepatitis B virus (HBV) infection is the major risk factor, accounting for 50% of HCC cases.[4] Other etiologies include infection by hepatitis C virus (HCV), chronic alcohol consumption, and NAFLD.[5] Although vaccinations (for HBV) and recent antiviral therapies (for HCV) have reduced viral HCC occurrence, HCC incidence continues to grow, mainly because of hazardous alcohol use and obesity/diabetes in Western countries.[6] The pathophysiology of HCC is a complex multistep process, with a heterogeneous mutational landscape and histological features.[7–10] Telomerase activation, induced by telomerase reverse transcriptase promoter mutations/rearrangements, is observed in 80% of HCC.[8,11] Next-generation sequencing has enabled the identification of the candidate cancer driver genes in HCC, such as *TP53* (28%–36%), *CTNNB1* (17%–37%), *AXIN1* (4%–14%), *ARID1A* (16.8%), and *ARID2* (5.6%), affecting cell-cycle control, Wnt/β-catenin pathway, and epigenetic machinery.[12–14]

Currently, only 25% of patients with HCC have at least one potential actionable mutation, whereas the main cancer driver genes remain undruggable.[15] Unfortunately, HCC does not respond to classical chemotherapies, and hepatic resection and liver transplantation are the main curative treatments.[16] Since 2010, systemic therapies based on tyrosine kinase inhibitors (TKIs) have improved patient outcomes. Sorafenib targets the RAF–MEK–ERK cascade and angiogenesis via vascular endothelial growth factor receptor (VEGFR) 2[17] and is used as front-line therapy but only confers a survival benefit of 2.8 months over placebo.[17] In 2018, the REFLECT phase 3 study reported the efficacy of lenvatinib, another TKI with more potent activity against VEGFRs and the FGFR family, with a slightly improved median overall survival compared with sorafenib (13.6 vs. 12.3 months).[18] Second-line treatment options for advanced HCC include other TKIs (regorafenib[19] and cabozantinib[20]) and ramucirumab, a monoclonal antibody specific for VEGFR2,[21] which has shown specific benefit for patients with high alpha-fetoprotein (AFP) serum concentrations after the failure of sorafenib (REACH study).[21]

Since 2017, other therapies to modulate the liver tumor microenvironment (TME) have emerged. Pembrolizumab and nivolumab, two immune-checkpoint inhibitors (ICIs) targeting programmed cell death-1 (PD-1), have been approved by the FDA as second-line treatments for advanced HCC (they failed to demonstrate significant superiority in overall survival over sorafenib).[22,23] A combination of nivolumab with ipilimumab, a monoclonal antibody targeting cytotoxic T-lymphocyte–associated protein 4 (CTLA4) has been approved as a second-line treatment for advanced HCC by the FDA.[24] In 2020, the atezolizumab (anti-programmed death-ligand 1 [PD-L1]) and bevacizumab (anti–vascular endothelial growth factor [VEGF]) combination was FDA approved as a first-line treatment for advanced HCC after showing superiority over sorafenib in phase 3 IMBRAVE-150 study (NCT03434379) with a 6.8-month median progression-free survival versus 4.3 months for sorafenib.[25] Still, despite these unprecedented and encouraging results, only 20%–30% of patients respond to immunotherapies, and so far biomarkers have failed to clearly elucidate the responding groups.[24,26] Taken together, there is an urgent need to better characterize the liver cancer microenvironment in order to design novel combination therapies that inhibit tumorigenesis and/or restore sensitivity to immunotherapy-resistant tumors. Also, the identification of biomarkers of response and resistance will improve patient selection for personalized treatment.

In this review, we provide updates about the role of the liver TME on HCC tumorigenesis. Additionally, we summarize the current knowledge about feasible treatments, new therapies, and current clinical trials targeting myeloid cells and lymphocytes. Although platelets are now recognized as important regulators of HCC and HCC TME,[27] given the unique nature of platelets (they are pieces of megakaryocytes that lack a nucleus), they are reviewed elsewhere.[28]

THE LIVER IMMUNE MICROENVIRONMENT

The liver contains a large reservoir of immune cells: neutrophils, monocytes, resident macrophages (Kupffer cells [KCs]), natural killer (NK) cells, natural killer T (NKT) cells, and liver-transiting and/or resident lymphocytes (B, $CD8^+$ T, $CD4^+$ T, and γδ T cells) (Figure 1).[29] The liver environment is highly tolerogenic toward gut-derived microbial metabolites in order to maintain a global homeostasis.[30,31] This immunotolerance results from continuous antigen presentation between liver-resident cells (hepatocytes, endothelial cells, KCs, and dendritic cells [DCs]) and peripheral leukocytes without costimulatory molecules, enabling the expansion of regulatory T cells (Treg) induced by KC-derived IL-10.[32] There is an overall balance between anti-inflammatory cytokines (IL-10, IL-13, and transforming growth factor β [TGF-β]) and proinflammatory ones (IL-2, IL-7, IL-12, IL-15, and interferon γ [IFN-γ]), maintaining the homeostasis.[33]

Chronic liver diseases lead to an upregulation of inflammatory signals and cause homeostatic disbalance associated with necroinflammation.[29] During chronic HBV infection, the load of circulating HBV or HBV-derived antigens promote T cell inactivity (exhaustion)

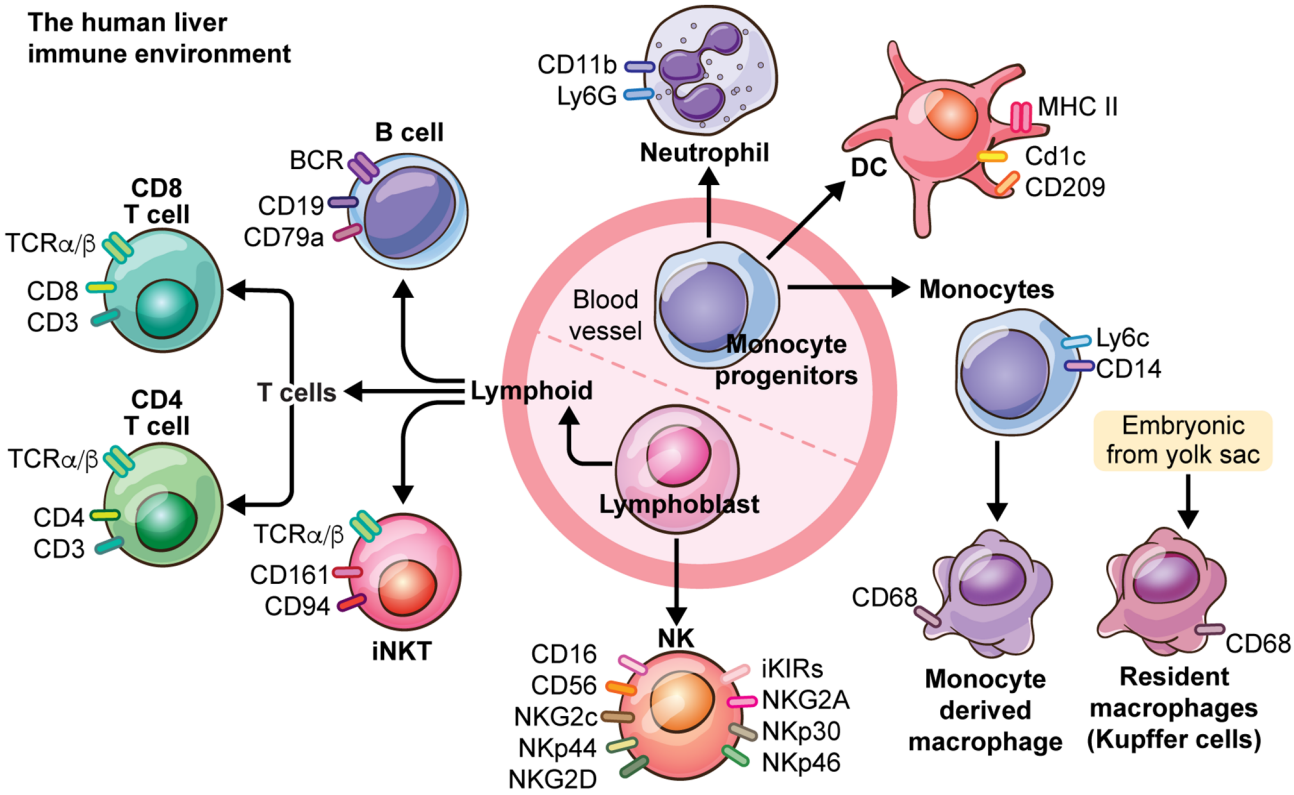

FIGURE 1 Understanding the liver microenvironment. Myeloid and lymphoid progenitors arise from hematopoietic stem cells via intermediate progenitors. In the steady-state, these progenitor cells supply cells to tissues for immune surveillance. Monocyte progenitors produce neutrophils, dendritic cells (DCs), and monocytes. Monocytes can be differentiated in macrophages in specific organs. In the liver, there are two categories of macrophages: the ones with an embryonic origin (the Kupffer cells) and the others differentiated from circulating monocytes (the monocyte-derived macrophages). In mice, specific markers to discriminate Kupffer cells have been discovered but in humans, they are still unknown. The lymphoblast lineage gives rise to natural killer (NK) cells and lymphocytes (invariant NKT), T cells, and B cells). Representative markers for each cell type are shown.

and subsequent death associated with a global weakening of immunity.[34–36] HCV avoids immune system recognition because of the high mutational rate[37] and through viral factors that counteract DNA sensors.[38–40] On the other hand, chronic alcohol consumption and NAFLD are characterized by sterile inflammation that amplifies proinflammatory signals and activation of monocytes, macrophages, and neutrophils.[41,42] For the last 10 years, immune cell involvement during chronic liver damage (CLD) has been intensively studied and shows a pivotal role in supporting disease progression. Moreover, there is increasing evidence implying a role of gut permeability, the microbiome, and microbial metabolites in CLD and HCC.[43,44] Recently, an ex vivo study has shown that bacterial extracts from patients with NAFLD-HCC elicit a T cell immunosuppressive environment with an attenuation of cytotoxic T cells.[45] Targeting the gut–microbiota–liver axis represents an exciting clinical opportunity; however, more work is needed to clinically and functionally validate the potential of targeting the microbiome for therapeutic purposes.

The majority of HCCs evolve in this chronic immunosuppressive necroinflamed environment. A decrease in T cell costimulatory factors associated with an increase in immune-checkpoint molecules results in impaired T cell effector functions.[46] In cancer, and especially in HCC, TME fuels the growth of cancer cells and forms a safe niche for them to counterbalance the activation of the immune system. Additionally, HCC tumors present an intermediate mutational load and a number of different immune evasion mechanisms.[47] Recent studies based on single-cell RNA sequencing (scRNAseq) have shown that HCC cells exhibit high interpatient variability, whereas the TME exhibits recurring gene expression signatures that are more uniform between patients.[48,49] These results suggest that strategies targeting the TME, such as immunotherapies, may be more effective in patients with HCC.

NEUTROPHILS: FRIENDS OR FOES?

Neutrophils are innate immune cells and one of the first cells to infiltrate a tissue during infection, injury, or tumorigenesis. High infiltration levels of tumor-associated neutrophils (TANs) in some solid human tumors have been shown to correlate with tumor growth, lymph node metastasis, and poor prognosis.[50] However, TANs come

in two different flavors: antitumorigenic (N1) or protumorigenic (N2).[51] Protumorigenic N2 TANs have the capacity to form decondensed chromatin embedded with granular proteins, called neutrophil extracellular traps (NETs), known to support tumor growth.[51] In HCC, CD66b$^+$ neutrophils enriched in the peritumoral area are correlated with decreased overall survival (n = 149).[52]

Multiple studies have highlighted the importance of cross-talk between tumor cells, TANs, and cancer-associated fibroblasts (CAFs) in influencing HCC progression. CAFs can suppress neutrophil function through the SDF1a/CXCR4/IL-6 pathway, which induces the expression of CD66b, PD-L1, CXCL8/IL-8, TNF, and CCL2, capable of suppressing the function and proliferation of T cells in vitro.[53] Secretion of cardiotrophin-like cytokine factor 1 (CLCF1) by CAFs can mediate tumoral expression of CXCL6 and TGF-β, responsible for neutrophil recruitment and "N2" polarization, respectively.[54] In human HCC, TANs acquired a protumoral N2 phenotype in the midstage to late stage of tumor progression in correlation with increased levels of CLCF1.[54] Indeed, CLCF1 may be a potential prognostic biomarker for HCC, and selective blockade of CLCF1 signaling could represent an effective therapy for patients with HCC (Figure 2).

N2 TANs can induce a stem cell–like phenotype in HCC cells.[55] Coculture of human TANs and HCC cell lines enhanced tumorigenic features such as proliferation, invasion, and death escape. Injection of HCC cells and TANs into non-obese diabetic-severe combined

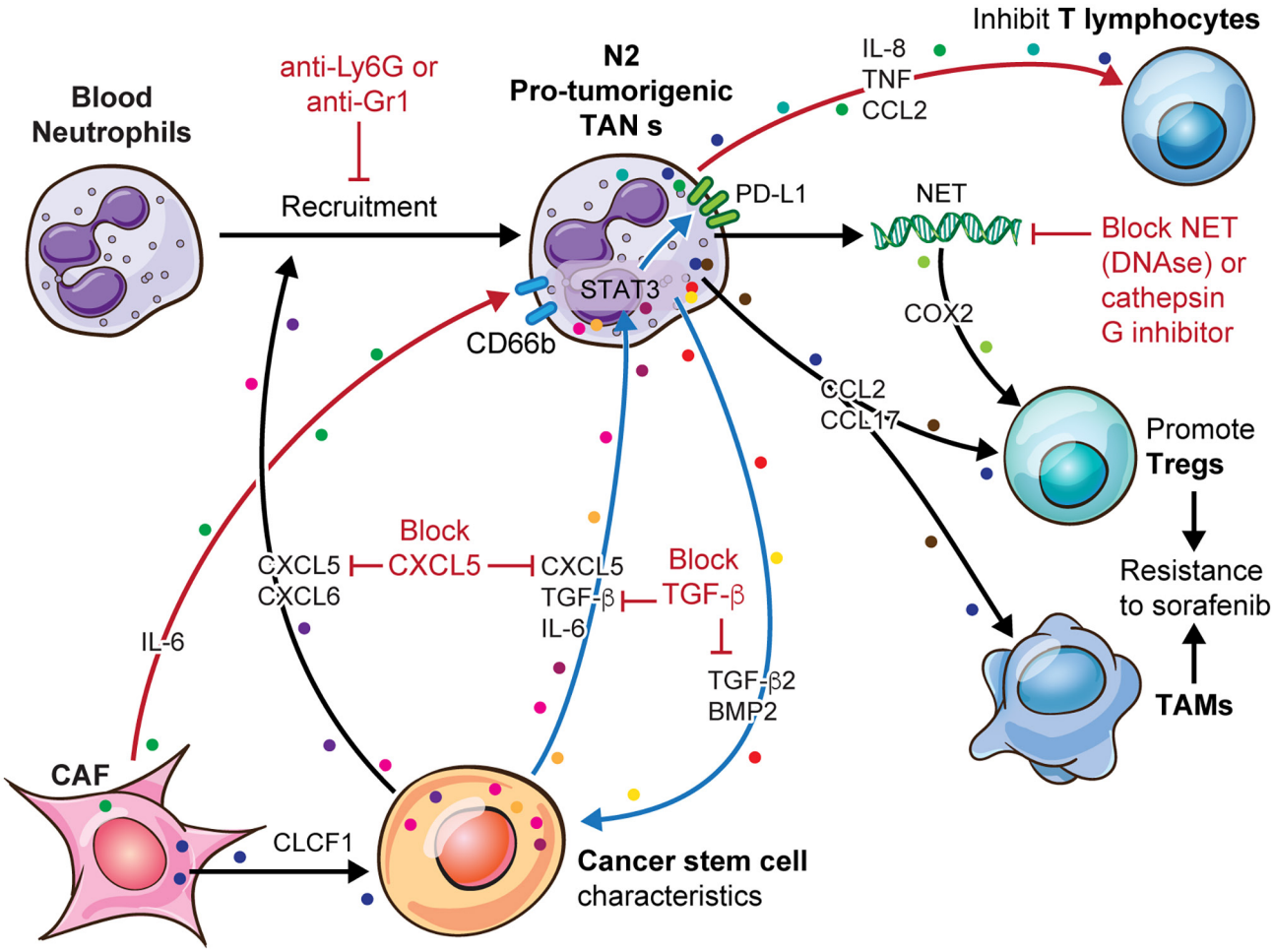

FIGURE 2 Neutrophils: friends or foes? Neutrophils are recruited at the tumoral site by the release of CXCL5 and CXCL6. Tumor-associated neutrophils (TANs) are turned into a protumorigenic (N2) phenotype by the tumor. These N2 TANs exhibit strong immunosuppressive functions including expression of programmed death-ligand 1 (PD-L1), release of immunosuppressive cytokines (IL-8, CCL2, CCL17), and NETosis. These features inhibit T cells, promote regulatory T cell (Treg) differentiation, and induce immunosuppressive tumor-associated macrophages (TAMs), leading to sorafenib resistance. There is an important dialogue between tumor cells, cancer-associated fibroblasts (CAFs), and TANs (blue and red arrows). Secretion of cardiotrophin-like cytokine factor 1 (CLCF1) by CAFs mediates tumoral expression of CXCL5, IL-6, and transforming growth factor β (TGF-β), responsible for neutrophil recruitment and "N2" polarization, respectively. Activation of STAT3 leads to PD-L1 expression associated with T cell inhibition and triggers a positive loop to amplify cancer stem cell characteristics with the release of TGF-β and bone morphogenetic protein 2 (BMP2). Strategies to inhibit the immunosuppressive function of TANs during hepatocellular carcinoma (HCC) could involve preventing their recruitment by using immunotherapies (anti-Ly6G or anti-Gr1), blocking the protumorigenic NETosis mechanism with DNase or cathepsin G inhibitor or to block some cytokine pathways such as CXC5 or TGF-β.

immunodeficiency mice (B and T cell-deficient) promoted HCC growth compared with HCC cells alone. Mechanistically, TANs secrete bone morphogenetic protein 2 and TGF-β2, leading to miR-301b-3p expression in cancer cells, which increases stem cell properties and the CXCL5-neutrophil chemoattractant as a positive feedback loop.[56] Multivariate analysis in three cohorts of patients with HCC (n = 919) indicated that CXCL5 overexpression, alone or combined with the presence of intratumoral neutrophils, was an independent prognostic indicator for overall survival.[56] CXCL5 secretion by HCC tumors is induced by the TGF-β and AXL pathways, suggesting that targeting these pathways might be an effective therapeutic strategy to combat HCC progression in patients who are TGF-β–positive.[57] These various studies highlight that CXCL5 inhibition may represent a potential therapeutic target to decrease TAN infiltration, which could be combined with tumor-targeted treatments. Interestingly, sorafenib treatment increases TAN infiltration in animal models and patients with HCC (n = 46) through the HIF1a/NF-κB/CXCL5 pathway and promotes their survival by inhibiting apoptosis.[58] In tumor-bearing mice, TANs induced resistance to sorafenib by recruiting macrophages and Treg cells though the secretion of CCL2 and CCL17. Indeed, human HCC tumors with low levels of $CCL2^+$ or $CCL17^+$ cells had longer survival times. TAN depletion with anti-Ly6G antibody in tumor-bearing mice inhibited tumor growth and neovascularization to a greater extent when combined with sorafenib than sorafenib alone.[58] Depletion of TANs with anti-Gr1 antibody in hepatoma-bearing mice reduced tumor size and microvessel density at the invading tumor edge[59] (Figure 2).

N2 TANs have also the capacity to form NETs, which can act to promote HCC development during NAFLD.[60] In the streptozoticin + high-fat-diet mouse model (NAFLD induced by neonatal streptozotocin and high-fat diet), inhibition of NET formation by DNase treatment did not affect the development of fatty liver but reduced tumor growth, mainly by reducing IL-6 levels. NETs link innate and adaptive immunity by promoting differentiation of $CD4^+$ T cells into Tregs via the modulation of the oxidative phosphorylation pathway.[61] Moreover, during late HCC stages, NETs can fuel invasiveness by promoting inflammation through activation of toll-like receptor 4/9-COX2.[62] In vivo treatment with anti-inflammatory drugs (aspirin) and DNase I, to block COX2 and the upstream pathway, was efficient in inhibiting HCC metastasis. Also, blocking NETs through the inhibition of cathepsin G, a serine protease released during NETosis, stops cancer cell invasion in vitro and decreased lung metastasis in mice.[63] Clinically, a higher density of cathepsin G protein in the peritumoral tissues was observed for patients with metastatic HCC than for those without metastasis. These findings implicate NETs in NASH-HCC and the late stages of HCC, suggesting that their elimination may reduce the initiation or progression of liver cancer (Figure 2).

TANs can also release many immunoregulatory and angiogenic factors capable of manipulating the TME or affecting cancer cells. Various studies have provided evidence that an elevated blood neutrophil-to-lymphocyte ratio is associated with poor outcomes in patients with HCC.[52,64–66] Altering TAN recruitment, migration, or activation could be an interesting therapeutic strategy, which is notably missing in clinical trials. This idea is supported by the finding that lenvatinib therapy increases TAN recruitment by inducing CXCL2 and CXCL5 secretion in the TME and forcing their polarization toward the N2 phenotype.[67] However, the combination of lenvatinib with a COX-2 inhibitor, celecoxib, reduced in vivo NET infiltration, and PD-1 exhausted T cells. Moreover, the TGF-β pathway has the capacity to promote N2 TANs but has also a pleiotropic role in multiple signaling pathways and can directly interfere with tumor development, favoring progression and driving immune evasion of cancer cells.[68] In HCC, TGF-β usually plays a dual role, acting as a tumor suppressor at early stages but contributing to tumor progression at late stages. Some clinical trials on targeting TGF-β or its receptors are being conducted in patients with HCC. Galunisertib/LY2157299, a novel TGF-β receptor 1 kinase inhibitor, is being investigated in phase II trials in combination with nivolumab (NCT02423343), sorafenib, or ramucirumab (NCT02240433, NCT02178358, and NCT01246986).

MONOCYTES: DR. JEKYLL OR MR. HYDE?

Monocytes are innate immune cells and play a dichotomous role in cancer. They are often recruited into the tumor through tumoral CCL2 production.[69] In HCC, different subsets of monocytes have been characterized along the tumorigenic process. During the early stages, recruited monocytes are able to kill tumor cells; however, tumors escaping immune surveillance hinder monocyte-induced death by reprogramming the monocytes into immune-suppressive cells. This has been demonstrated in a humanized murine model transplanted with human HCC cell lines, in which injection of $CD14^+$ monocytes from patients with early HCC was able to kill transplanted HepG2 cells. However, $CD14^+$ monocytes from the same patients after progression to the advanced stage lacked antitumor activity and expressed PD-L1/2, which counterbalanced the antitumor activity of $CD8^+$ T cells against HepG2 and Huh7 cells.[70] Finally, the overall survival of patients with PD-$L1/2^+$ $CD14^+$ monocytes was shorter than those with other $CD14^+$ cells (n = 87). These PD-$L1/2^+$ $CD14^+$ monocytes also expressed immune-suppressive cytokines such as IL-10 and CCL1.[71] Tumor-educated

$CCR1^+CD14^+$ monocytes express PD-L1, B7-H3, and TIM3, and upregulate tolerogenic metabolic enzymes, promoting angiogenesis and metastasis.[72] Analysis of chemokine expression in HCC cell lines and tissues identified CCL15 as a main recruiter of this $CCR1^+CD14^+$ subset to the tumor invasive margin,[72] and in two cohorts of patients (n = 360 and n = 253), those with high CCL15 levels had a worse prognosis. Blocking CCL15–CCR1 axis significantly inhibited orthotopic in vivo tumor growth and lung metastasis and could be an interesting clinical strategy to circumvent the recruitment of $CCR1^+CD14^+$ monocytes. Importantly, CCL15 can be measured in the blood and could be used as a biomarker[73] (Figure 3).

Another therapeutic option could be to avoid the generation of protumorigenic monocytes by inhibiting essential metabolic enzymes. A glycolytic switch in peritumoral $CD86^+HLA\text{-}DR^+$ monocytes has been reported to regulate HCC TME in patients.[74] Mechanistically, the PFKFB3 glycolytic enzyme is induced during the monocytic metabolic switch that leads to PD-L1 expression, thus contributing to $CD8^+$ T cell inactivation, immune evasion, and disease progression in human HCC.[74] The same monocyte population can also recruit neutrophils within the tumor via CXCL2/CXCL8, which in return produce the prometastasic factor oncostatin M, favoring disease progression[75] (Figure 3). The level of oncostatin M was found to positively correlate with metastasis in patients with HCC.[75]

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous subset of myeloid cells that have been shown to inhibit T-cell responses in cancer and HCC. Two different populations of MDSC have been described: monocytic (M-MDSC-$CD14^+$) and polymorphonuclear (PMN-MDSC-$CD11b^+CD33^+HLA\text{-}DR^-$). Both share phenotypic and morphologic features with monocytes and neutrophils, respectively.[76] An increase of the specific M-MDSC subset has been observed in the peripheral blood (n = 111) and tumor (n = 12) of patients with HCC.[77] This population was able to induce $CD4^+CD25^+Foxp3^+$ Tregs and inhibit autologous NK cell cytotoxicity by NKp30-dependent cell contact during in vitro coculture.[77,78] In tumors expressing the oncogenic cell cycle–related kinase (CCRK), MDSC accumulation was observed within the tumor and blood of patients with HCC, and high CCRK/IL-6/CD11b/CD33 expression was associated with the worst prognosis.[79] Preclinical HCC mouse models have demonstrated that CCRK signaling coordinates the establishment of an immunosuppressive TME by

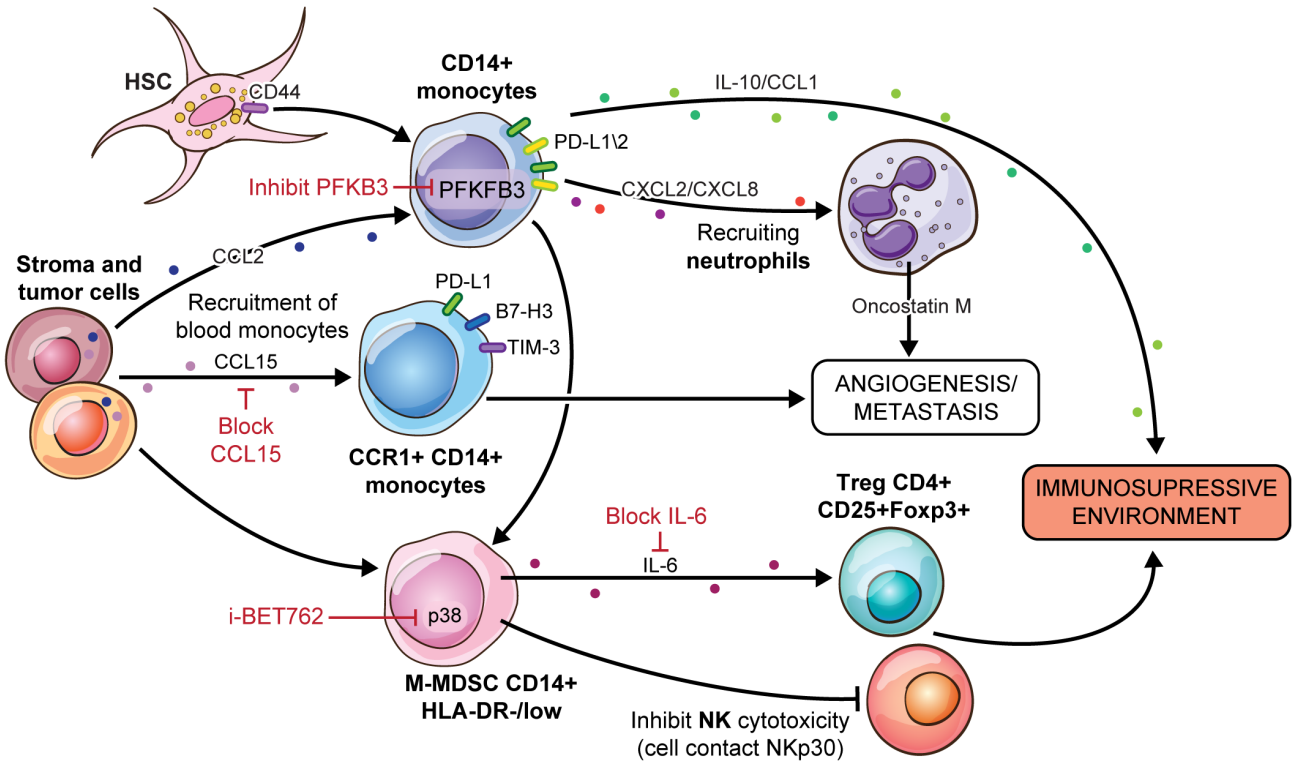

FIGURE 3 Monocytes: Dr. Jekyll or Mr. Hyde? Monocytes are recruited into the tumor site by the release of tumoral and stromal chemokines, such as CCL2 and CCL15. Monocytes can be polarized into different subtypes such as $CD14^+$, $CCR1^+CD14^+$, and myeloid-derived suppressor cells (MDSCs). All of these subtypes promote a strong immunosuppressive environment with expression of immune-checkpoint inhibitors (programmed death-ligand 1/2 (PD-L1/2), B7-H3, TIM3) and cytokines (IL-10, CXCL2, CXCL8), inhibiting natural killer (NK) cytotoxicity and inducing regulatory T cells (Tregs). They also interact with neutrophils to promote tumor invasiveness though oncostatin M pathway. Ways to control tumorigenesis through monocytes could be to prevent their recruitment to the tumor by inhibiting the CCL15 pathway, to block their polarization by inhibiting the p38 pathway, or to repress the IL-6 pathway in order to prevent Treg formation.

recruiting MDSCs, which can be counteracted by targeting CCRK or IL-6. Hepatic stellate cells (HSCs) can also induce M-MDSCs from circulating $CD14^+HLA\text{-}DR^{high}$ monocytes through a CD44-dependent contact and an unknown partner.[80] In a cohort of patients (n = 31), $CD33^+CD\text{-}11b^+$-MDSCs were enriched in the tumor-surrounding fibrotic livers.[81] The authors also found, in a human HSC-PBMC coculture system that activated HSC cells, release factors to activate monocyte-intrinsic p38 signaling, leading to M-MDSC–specific gene expression. Activation of p38 in MDSCs was requisite for the recruitment of C/EBPβ and p300 to the chromatin, thus promoting H3K27 acetylation and BRD4 binding at enhancers. In PBMCs derived from patients with HCC, blocking the enhancers with i-BET762 led to M-MDSC reduction. Furthermore, treatment of a CCl_4-induced fibrotic HCC model with i-BET762 and anti-PD-L1 improved the antitumor activity by reducing M-MDSC and increasing $CD8^+$T cells, which improved long-term survival. This combination was well tolerated by the mice and could represent an emerging strategy to treat patients. i-BET762 (Molibresib/GSK525762) is currently under investigation in phase I/II clinical trials (NCT02964507/NCT03150056) to treat advanced or metastatic breast cancer and castration-resistant prostate cancer (Figure 3). Profiling of monocytes may provide a diagnostic or prognostic marker for HCC. Furthermore, the development of therapies targeting monocyte recruitment or differentiation could be an interesting way to alter neutrophil recruitment and $CD8^+$ T cell inhibition.

MACROPHAGES: THE TOUGH ONES

In homeostasis, monocyte-derived cells develop into liver DCs or macrophages. Additionally, the liver contains a pool of resident macrophages, the KCs, which originate from yolk sac–derived precursors during embryogenesis.[82] KCs are activated by danger signals and then promote chronic liver inflammation by inducing the recruitment of immune cells, including monocytes.[82] According to their environmental stimuli, macrophages can be divided into two main subtypes, M1 and M2. M1 macrophages are induced by microbial components or by proinflammatory cytokines (TNF, IFN-γ, toll-like receptor [TLR]) and exert proinflammatory functions by releasing nitric oxide, reactive oxygen species, and proinflammatory cytokines IL-1, IL-6, IL-12, TNF-α, CXCL5, and CXCL8/10. In contrast, M2 macrophages are polarized by IL-4, IL-10, and IL-13 and glucocorticoids, and exert immunosuppressive functions to promote tissue repair. The CD68 marker is commonly used for liver tumor-associated macrophage (TAM) location and distribution, and the expression levels of CD86 (M1), CD163 (M2), and CD206 (M2) are accepted to distinguish between M1 and M2 macrophages in vitro.[83] However, the M1/M2 nomenclature has become controversial because of the existence of many distinct polarization phenotypes that have been seen in tissues and driven by a number of different stimuli.[82,84] Within the M2 subclass, there are several subtypes (M2a, M2b, M2c, and M2d) characterized according to their activation stimuli.[85] However, these subclasses have been difficult to identify in vivo because of the wide spectrum of activation states and markers.[86]

Macrophages are major components of the HCC TME and present several tumor-promoting roles including immune suppression, metastasis, angiogenesis, maintenance of cancer cell stemness, and drug resistance.[87,88] Different studies have reported that high levels of TAMs, especially in the peritumoral area, are associated with poor prognosis in patients with HCC.[89–92] High TAM infiltration within the tumor or at the marginal site can also predict poor prognosis after tumor resection.[93] The analysis of $CD68^+$-TAMs in a cirrhotic HBV-positive cohort of patients (n = 137) has shown that marginal macrophage density was associated with vascular invasion, tumor multiplicity, and fibrous capsule formation. Also, a dysregulated balance toward $CD206^+$ M2 macrophages has been associated with an aggressive phenotype with advanced tumor-node-metastasis stage, poor overall survival, and decreased time to recurrence in an HBV^+-associated cirrhosis/HCC cohort (n = 253).[94] The density of TAMs has also been correlated with resistance to transarterial chemoembolization in a small cohort of patients (n = 26).[95] Consequently, immunotherapies targeting TAMs have emerged as a promising approach to treating patients with HCC and can be divided into different mechanisms, including inhibition of monocyte recruitment, elimination of preexisting TAMs in the tumor tissue, remodeling TAM polarization, promotion of phagocytosis, and neutralization of protumorigenic factors secreted by TAMs (Figure 4).

Some studies have investigated the mechanism of monocyte recruitment and TAM accumulation in HCC. Mitochondrial fission occurring in HCC cells has been correlated with the infiltration of $CD163^+$-TAMs (n = 69).[96] Indeed, fission induces cytosolic mitochondrial DNA in HCC cells, which activates the TLR9/NF-κB pathway, which in turn increases the production of CCL2, a TAM chemoattractant. Blockade of the CCL2/CCR2 signaling pathway seems a promising approach to suppress monocyte/TAM recruitment and M2 polarization and thus enable activation of an antitumor $CD8^+$ T cell response.[97] Moreover, CCL2 is overexpressed in human HCCs and is associated with poor prognosis. A combination of Nivolumab (anti-PD-1) with a CCR2/5-inhibitor is currently being tested in phase II clinical trial (NCT04123379). The osteopontin/CSF1/CSF1R pathway is another factor accounting for the accumulation of TAMs and failure of ICIs.[98] Osteopontin expression

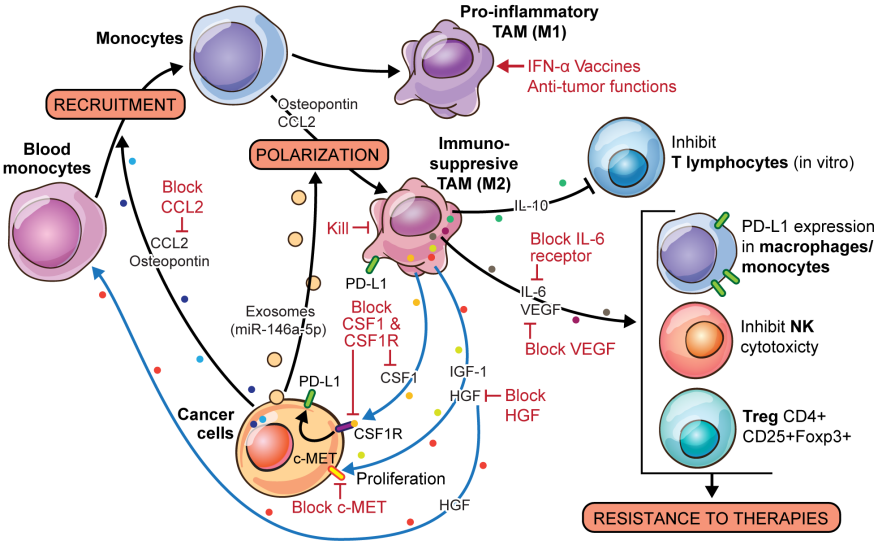

FIGURE 4 Macrophages: the tough ones. Macrophage-derived monocytes can be recruited by CCL2 to the tumor site. The proinflammatory (M1)/anti-inflammatory (M2) nomenclature is controversial, but it is still used to describe macrophage activity. Hepatocellular carcinomas (HCCs) educate tumor-associated macrophages (TAMs) to become immunosuppressive, notably through the secretion of CCL2 and osteopontin (SPP1), and the release of extracellular vesicles. CSF1 expression by macrophages leads to tumoral programmed death-ligand 1 (PD-L1) expression and increases the immunosuppressive environment. Moreover, M2 cells release cytokines such as IL-10, IL-6, and vascular endothelial growth factor (VEGF), leading to T and natural killer (NK) cell inhibition, macrophage immunosuppression, and regulatory T cell (Treg) differentiation responsible for resistance to therapies. Also, TAMs secrete hepatocyte growth factor (HGF) and IGF-1, leading to tumor cell proliferation and monocyte recruitment. Strategies to block their recruitment (anti-CCL2) or prevent their protumorigenic functions (inhibition of VEGF, HGF, c-MET or IL-6 pathway, Interferon α [IFN-α] vaccine) could lead to reduced tumor burden.

is positively associated with PD-L1 expression as well as TAM infiltration in human HCC tumors. In some HCC murine models, osteopontin facilitates chemotactic migration and M2 polarization and promotes the expression of PD-L1 in HCC cells via activation of the CSF1 receptor. Consequently, CSF1/CSF1R blockade may enhance the efficacy of anti-PD-1/L1 immunotherapies by hijacking TAMs.[98] The CSF-1R inhibitor PLX3397 was able to suppress tumor growth in tumor-bearing mice by promoting a polarization shift toward the M1 phenotype, a decrease in MDSCs, and $CD4^+$ T cells in tumors counterbalanced with an increased number of $CD8^+$ T cell.[99] A phase I study is currently testing an MET/CSF1R/SRC inhibitor (TPX-0022) in patients with advanced solid tumors including HCC (NCT03993873) (Figure 4).

The suppression of TAMs is a potential strategy to treat HCC. Sorafenib in combination with TAM-depleting agents (clodrolip or zoledronic acid) induces antimetastatic and antiangiogenic effects.[100] Interestingly, Sorafenib treatment led to a higher presence of F4/80 and CD11b-positive cells in the blood and within the tumor. In a nude mouse model bearing HCC cell lines, sorafenib induced tumor necrosis responsible for HNF-1α secretion and chemoattraction of TAMs. Combination with antimacrophage drugs reduced VEGF and led to the enhanced inhibition of tumor growth and lung metastasis. Moreover, the combination of Sorafenib and zoledronic acid synergized to decrease $CXCR4^+$ vascular density in the tumor.[101] Indeed, similar preclinical observations were made in rat HCC models treated with TACE and zoledronic acid.[102] Interestingly, zoledronic acid has been widely used in the prevention of bone metastasis and reduction of skeletal tumor burden in many kinds of cancer by inhibiting osteoclast activity, and it is well tolerated in patients.[103,104]

The reeducation of TAMs could be a potential alternative strategy to treat HCC. Indeed, miR-146a-5p–embedded exosomes secreted from HCC cells can educate macrophages and promote M2 polarization to enable protumoral effects, notably by inducing PD-L1 upregulation and T cell exhaustion.[105] Recently, extracellular vesicles showed a role in monocyte differentiation through activation of the glycolysis.[106] M2 macrophages, polarized from the monocytic cell line THP-1, confer in vitro hepatoma resistance to sorafenib through hepatocyte growth factor (HGF)/c-MET signaling. HGF also increases monocytes' recruitment from the blood, creating a feed-forward loop. In tumor-bearing mice, M2 TAMs were accumulated in sorafenib-resistant tumors more than in sorafenib-sensitive tumors and produced an abundant amount of HGF. These new insights brought a rationale for the use of c-MET/HGF inhibitors (cabozantinib or tepotinib) alone or in combination to improve systemic therapeutic efficacy. Tepotinib is currently tested in phase Ib/2 for patients with advanced HCC, whereas the combination of cabozantinib with atezolizumab is ongoing in patients with advanced HCC in a phase III clinical trial

(NCT02115373-NCT03755791).[107] TAM polarization toward the M1-like phenotype can also be induced by IFN-α and has a synergistic effect with sorafenib inhibiting HCC growth and metastasis in an orthotopic HCC implantation model.[108] Another strategy could be to skew TAM polarization from M2 into M1 with vaccines, which have shown a synergistic antitumor effect with anti-PD-1 immunotherapy in an HCC-bearing mouse model.[109]

Neutralization of protumorigenic factors secreted by TAMs is another possible strategy to employ. Elevation of IL-6 in the serum of patients with HCC is linked to the expression of PD-L1 in HCC monocytes and macrophages.[110] Notably, IL-6 expression is correlated with stemness and tumor progression, and this cytokine can be produced by TAMs and MDSCs.[111] Tocilizumab, an FDA-approved humanized anti–IL-6 receptor antibody for rheumatoid arthritis treatment, was able to inhibit liver tumorigenesis. Interestingly, a recruiting clinical trial (NCT04524871) will try to evaluate the efficacy and safety of multiple immunotherapy-based treatment combinations (atezolizumab, bevacizumab, and tocilizumab) in patients with advanced liver cancers. The anti–IL-6 antibody siltuximab could be another method because it is approved by the FDA for the idiopathic multicentric Castleman disease.[112,113]

Recently, by combining the single-cell atlases from human fetal livers and HCCs (n = 14), a new TAM subset has been discovered to secrete VEGF and promote oncofetal reprogramming of the TME and severity of HCCs.[49] These fetal liver–associated $CD163^+$-TAMs were able to interact with immune cells through immune-checkpoint receptors and other ligands (CD40LG/CD40, CD28/CD86, SIRPA/CD47, and CD86/CTLA4). Interestingly, the VEGF pathway promotes immune suppression in different ways, including inhibition of antigen-presenting cells and activation of suppressive MDSCs, TAMs, and Treg cells, providing a rationale for combining ICIs with antiangiogenic agents.[114] The IMBrave150 HCC clinical trial (NCT03434379) combined VEGF inhibitor bevacizumab with atezolizumab and has recently shown an outcome improvement in comparison with sorafenib monotherapy that led to its FDA approval.[115,116] By suppressing immunosuppressive cells and increasing DC maturation and cytotoxic T cell activity, VEGF inhibitors could allow the switch from cold tumors to hot tumors.[117] However, high doses or long-term therapy with anti-VEGF agents can lead to an excessive reduction of vessels and promote hypoxia, highlighting the challenges of using anti-VEGF therapy.[117] Of note, regorafenib and sorafenib have similar targets but regorafenib has a more potent antiangiogenic effect.[118]

The role of macrophages and TAMs in HCC represents a double-edged sword because these cells have both antitumor and protumor functions. Targeting these populations or reeducating them by forcing a phenotypic switch could represent a potential therapeutic treatment. Also, improving the phagocytosis capacity of macrophages could lead to a reduced tumor burden as demonstrated in other solid cancers.[119]

DCs GET T CELLS ROLLING

DCs constitute an essential link between innate and adaptive immunity because they orchestrate the antigen presentation, leading to activation and differentiation of T cells. In contrast to macrophages, DCs have migratory properties and present antigen to T cells in the tissue-draining lymph nodes[120]; however, many DC–T cell interactions also occur in the liver.[121] $CD8^+$ T-cell activation depends on the previous activation of a DC by $CD4^+$ T helper (Th) cells.[122] A fully functioning immune synapse is defined by three regulated steps.[123] First, DCs must present the antigen on MHC class II molecules to $CD4^+$ Th cells and on MHC class I molecules to $CD8^+$ T cells. Secondly, the interaction of costimulatory molecules of the immunoglobulin superfamily (CD80-CD86/CD28) and the TNF superfamily (CD40L/CD40, 4-1BBL/4-1BB, CD27/CD70, CD30L/CD30, and HVEM/LIGHT) must occur to trigger the production of cytokines (third step) that stimulate $CD8^+$ T cell expansion and differentiation, such as IL-12 and type I IFN (Figure 5A). The $CD8^+$ T cell licensing can be facilitated by $CD4^+$ Th cells ("classical licensing") or by NKT cells ("alternative licensing").[123] Interference at one of these three steps will lead to a dysfunctional adaptative response. Therefore, one of the main mechanisms used by cancer cells to escape immune surveillance is the disruption of this immune synapse, commonly through the expression of inhibitory ligands preventing T cell activation.[124] T-cell exhaustion is defined as an impaired T-cell capacity to proliferate and secrete cytokines and can be achieved with the overexpression of inhibitory immune-checkpoint receptors (e.g., PD-1, CTLA4; lymphocyte-activating 3 [LAG3]; hepatitis A virus cellular receptor 2, TIM3).[125] As described before, inhibitory immune-checkpoint ligands are not only expressed by HCC tumor cells but also by myeloid cells such as DCs, TAMs, and neutrophils.

There is a great heterogeneity of DC populations depending on their developmental lineage, differentiation stage, and physiological and pathological microenvironment. They are usually grouped as conventional DCs (cDCs), plasmacytoid DCs (pDCs; $CD303^+CD304^+$, secreting type I IFN), and inflammatory DCs ($SIGN^+$, differentiated from monocytes in chronically inflamed environments, inducing Th-17 differentiation).[126] cDCs, also known as myeloid DCs, can be further divided into two categories: $CD141^+/CD14^-$ type 1 cDCs (cDC1) and $CD1c^+/CD14^-$ type 2 cDCs (cDC2).

In the peripheral blood of patients with HCC, several studies have observed a decrease in circulating

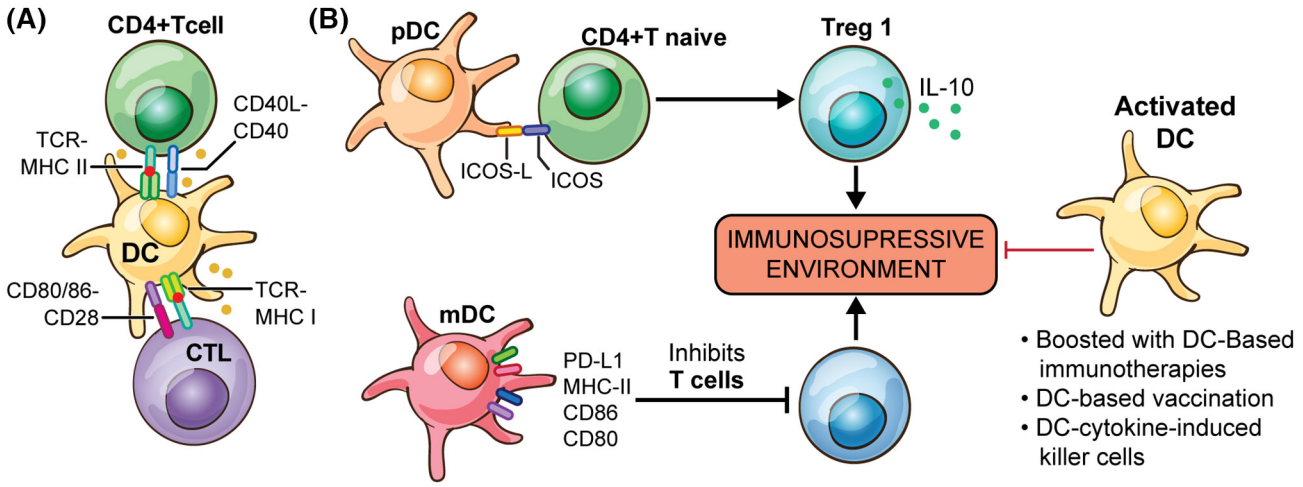

FIGURE 5 Dendritic cells get the T cells rolling. (A) The dendritic cell (DC)–T cell synapse is defined by three regulated steps: first, DCs present the antigen on MHC class II molecules to $CD4^+$ T helper cells and on MHC class I molecules to $CD8^+$ T cells. Then, interaction of costimulatory molecules of the immunoglobulin superfamily (CD80 and CD86, which bind to CD28 on T cells) and the TNF superfamily (CD40L/CD40, 4-1BBL/4-1BB, CD27/CD70, CD30L/CD30, and HVEM/LIGHT) occur to trigger cytokine release and T cell activation and differentiation. (B) Plasmacytoid DCs (pDCs) can induce type 1 regulatory T cells (Tr1) from naive $CD4^+$ T cells through ICOS-L/ICOS interaction, allowing production of IL-10 from regulatory T cells (Tregs). $LAMP3^+$ DCs (mDCs) can also express inhibitory ligands such as programmed death-ligand 1 (PD-L1), Gal9 (ligand of TIM3), MHC-II (for lymphocyte-activating 3 [LAG3]), and CD86 and CD80 (for cytotoxic T-lymphocyte–associated protein 4 [CTLA4]). These mechanisms lead to an immunosuppressive environment. Artificial activation of DCs (immunotherapy, vaccination…) could lead to an immunocompetent environment favoring an antitumor response.

pDCs and cDCs and a lower expression of costimulatory molecules on these DCs, as compared with healthy controls, which was inversely correlated with IL-10 concentration.[127,128] $CD303^+$ pDCs were found accumulated within human HCC tumors (n = 39) and localized with type 1 Tregs (Tr1; $P3^-CD49^+LAG\text{-}3^+$), and associated with poor prognosis in three cohorts of patients (n = 1065).[129,130] In fact, high numbers of $BDCA2^+$pDCs within tumors correlated with high AFP levels, advanced tumor-node-metastasis stage, and increased tumoral infiltration of Tregs and IL-17–producing cells. In vitro, pDCs were able to induce Tr1 from naive $CD4^+$ T cells through ICOS-L/ICOS interaction, allowing the production of Tr1 IL-10 (Figure 5B). Furthermore, tumor cDCs were found to express inhibitory ligands such as PD-L1, Gal9 (ligand of TIM3), MHC-II (for LAG3), and CD86 and CD80 (for CTLA4) in human HCC (n = 59).[131] scRNAseq is currently helping to find new subsets and markers of DCs that highlight DC heterogeneity.[132] In a small cohort of patients with HCC (n = 18), tumoral DCs expressed high levels of MHC-II molecules but low levels of MHC-I molecules compared with PBMC DCs.[133] cDC1 and cDC2 were distinguished but only cDC2 were accumulated in the tumor and were potentially able to present specific antigens to $CD4^+$ T cells.[133] Also, a new cDC class (cDC3) was identified in patients with HCC, with the expression of *CCL19*, *LAMP3*, and *CCR7*.[133] More recently, scRNAseq on 16 tumors from patients with treatment-naive liver cancer uncovered three DC subsets enriched within the tumors: DC-c1-CD1C, DC-c3-CLEC9A, and DC-c4-LAMP3.[134] The DC-c4 $LAMP3^+$ subset, enriched in the HCC core, expressed maturation and migration markers (LAMP3, CD80, CD83, CCR7) but also PD-L1/2, and was predicted to bind to and inhibit $PD\text{-}1^+$T cells. Analysis of liver HCC TCGA data indicated a strong correlation between the $LAMP3^+$DC signature and exhaustion/regulation of T cells (Figure 5B).

Strategies augmenting the $DC/CD8^+$ T cell dialogue have been developed, such as adoptive immunotherapy and DC-based vaccines, to attempt to restore an efficient antitumor response. A meta-analysis conducted on clinical trials (n = 1276 patients, 19 trials) highlighted that DC-based immunotherapies led to a better outcome, enhanced the $CD4^+$ $T/CD8^+$ T ratio, and were safe for patients.[135] DCs-OK432 generated with a streptococcus-derived anticancer immunotherapeutic agent (OK432) were able to produce large amounts of Th1 cytokines (IL-12 and IFN-γ) and enhance cytotoxic T cell activity through CD40/CD40L interactions.[136] These DCs-OK432 can be developed from harvested patient monocytes, stimulated with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), and then with OK432.[137,138] Patients with HCC (n = 13) who were administered DCs-OK432 during transcatheter hepatic arterial embolization presented a prolonged recurrence-free survival.[137,138]

DC-based vaccination is another therapeutic approach based on the ex vivo exposure of monocyte-derived DCs to tumor antigens (tumor lysates or tumor-associated antigens [TAAs]) with concomitant stimulatory signals (GM-CSF, IL-4, TNF-α), to trigger antitumor responses when introduced back into the

patients.[139] Some years ago, DC vaccines failed to show an improved outcome for patients with HCC despite their safety and tolerability.[140–142] However, AFP-pulsed DCs were able to stimulate specific cytotoxic T lymphocytes toward AFP-producing HCC cells.[143,144] Recently, the combination of DC vaccine with ICIs has shown promising results.[145,146] In preestablished in vivo subcutaneous and orthotopic HCCs, AFP-DCs alone induced a significant reduction and a slight delay of tumor growth, whereas combination of AFP-DCs and CD40L-expressing DCs had a synergistic effect associated with an enhanced Th1-cytokine level, tumor infiltration of cytotoxic T lymphocytes, and tumor apoptosis.[147] Also, an orthotopic HCC mouse model treated with a DC vaccine in combination with a PD-1 inhibitor showed longer overall survival than monotherapy and led to a greater reduction of tumor volume by inducing apoptosis.[145] Data from clinical trials have shown that the most promising strategy is the combination with ICIs and could represent a potential treatment for melanoma and prostate cancers.[148]

A meta-analysis conducted on 22 studies involving 3756 patients has reported that DC-based immunotherapies with cytokine-induced killer cells (CIKs) in combination with various standard-of-care treatments can improve patient prognosis.[149] DC-CIK therapies refer to mononuclear cells harvested from patients and activated, amplified, and modified to mediate activation of CIKs, mainly $CD3^+CD56^+$ type II killer T lymphocytes (NKTs).[150] A DC-CIK therapy has been recently studied in a small cohort of patients with HCC (n = 36), in which DC cells loaded with CD24 (a marker of cancer stem cells) were able to decrease serum Treg concentration and lead to an increase in $CD3^+$, $CD4^+$, and $CD56^+$ markers, and improved survival.[151] This study highlights that DC-CIK therapies can modify immune balance and have potential therapeutic benefits in the long-term control of tumor progression.

Dysfunction of DC-mediated antigen cross-presentation and subsequent T cell suppression is a central mechanism of immune escape in tumors. Adoptive DC immunotherapies and DC-based vaccines are promising immunological strategies to enhance the DC/T cell dialogue and restore an efficient antitumor response to HCC (Figure 5B). However, it seems important to highlight that these therapies are based on an optimal maturation status of DCs, the choice of tumor antigens, the dose, and the route of administration.

NK CELLS SHOOT TO KILL

NK cells are a granular $CD3^-$ lymphocyte population that triggers direct innate immune reactions against pathogens and malignant cells.[152] They represent about 10% of the lymphocytes in human peripheral blood and are subdivided into two main populations: the immune-modulator ($CD56^{bright}/CD16^-$) subset and the cytotoxic ($CD56^{dim}/CD16^+$) subset. NK cells express inhibitory receptors such as inhibitory killer immunoglobulin-like receptors and the C-type lectin-like receptor NKG2A, binding MHC-I and the nonclassical MHC-I complex, HLA-E, respectively. The absence of these ligands on a cell engages activating NK receptors (NKp30, NKp46, NKp44, NKG2D, and NKG2C) that bind their putative ligands expressed on infected or tumor-transformed cells[152] (Figure 6A). High cytotoxic activity of peripheral blood NK cells positively correlates with reduced cancer risk.[153] DCs also play an important role in NK cell–mediated antitumor activity through both direct contacts as well as a plethora of inflammatory cytokines (IL-15, IL-12, IL-18) that foster NK cell proliferation, stimulate cytokine production, and induce cytolytic activity.[154]

Liver NK cells, particularly those enriched with the immune-modulator $CD56^{bright}$ ($CXCR6^+CCR5^+CD69^+$) subset, represent the most abundant population among the intrahepatic lymphocytes (up to 50%), whereas HCC-infiltrating NK cells express memory-like NK cell markers such as KLRC1 and KLRC2.[49] The presence of a high number of NK and $CD8^+$ T cells predicts a better outcome in the early stage of the disease and correlates positively with apoptotic tumor cells in human HCC.[155] Nonetheless, NK cells usually lose antitumor properties. The frequency of both peripheral blood and liver $CD56^{dim}$ NK cells is decreased whereas immune-modulator NKs are expanded in patients with stage III HCC (n = 110).[156] Intratumoral $CD56^{dim}$ NK cells express immune-checkpoint molecules PD-1 and NKG2A, which are associated with poor prognosis (n = 207)[157,158] (Figure 6A). Blocking IL-10 specifically inhibited NKG2A expression in NK cells in vitro, suggesting that targeting IL-10 could restore immunity by reversing NK cell exhaustion.[158] Moreover, MDSCs and tumor cells are responsible for NK inhibition through NKp30 in patients.[78,159] Monocytes from HCC tissues incubated with circulating NK cells led to a transient activation and then strong exhaustion associated with cell death triggered through a CD48/2B4 dialogue.[160] Finally, sorafenib induces NK cell activation, in vitro and in tumor-bearing mice, through cytokines secreted by macrophages (IL-12, IL-18, IL-1β), suggesting that reinforcing NK functions could be a mechanism of sorafenib's antitumoral effect.[161,162] Overall, NK cells lose antitumor function in HCC because of a disbalance between their different subsets, prompting the immune-modulator subset to hijack immunosurveillance. These studies have opened new avenues for NK-mediated therapeutic strategies, including adoptive transfer of activated NK cells, administration of molecules that activate NK cell function, and the use of antibodies that block the interaction between inhibitory receptors and their ligands.[163–165]

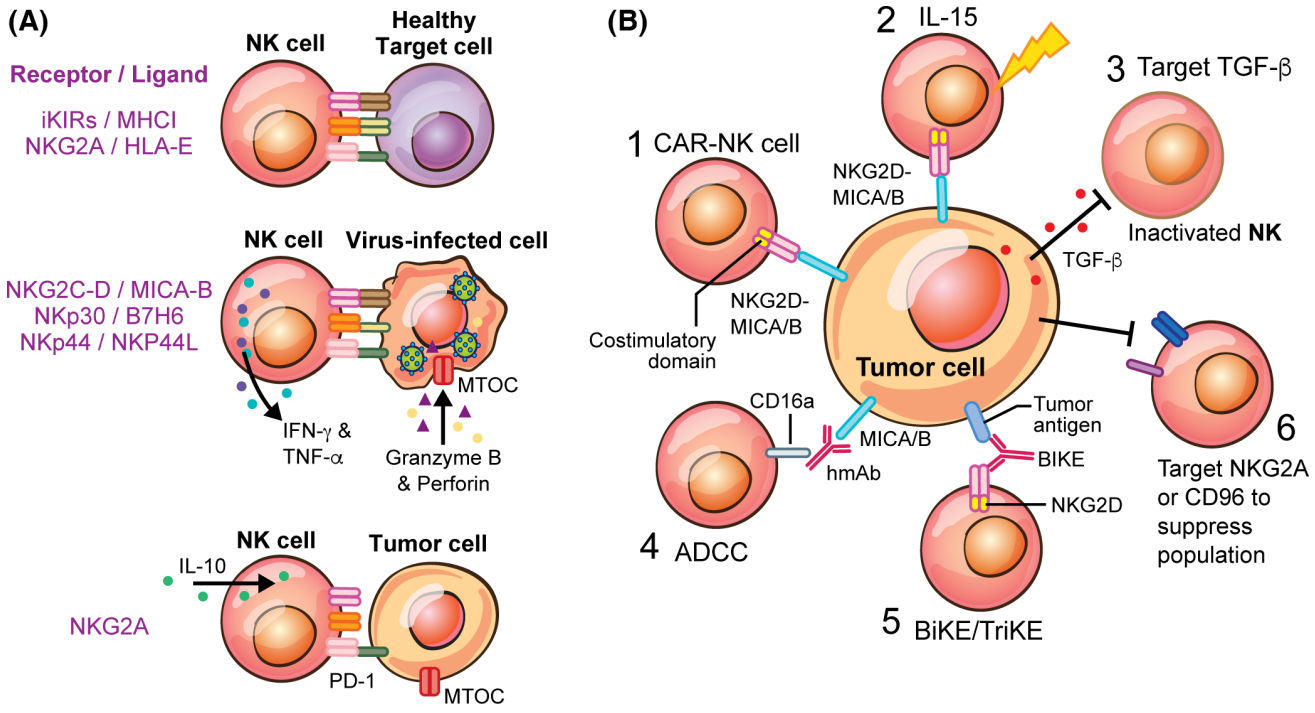

FIGURE 6 Natural killer (NK) cells shoot to kill. (A) NK cells express inhibitory receptors such as the inhibitory killer immunoglobulin-like receptors (iKIRs) and the C-type lectin-like receptor NKG2A, binding MHC-I and the nonclassical MHC-I complex, HLA-E, respectively. Healthy cells inhibit NK cells by binding to NKG2A and iKIRs. However, absence of these ligands on a cell engages activating NK receptors (NKG2C/D, NKp30, and NKp44) that bind their ligands (MICA-B, B7H6, NKP46L, respectively) expressed on infected or tumor-transformed cells. After binding, perforin and granzyme B are released and kill the cell. However, an immunosuppressive tumor microenvironment—through IL-10 signaling—leads to the expression of NKG2A in NK cells. NKG2A associated to the expression of programmed cell death-1 (PD-1) induces NK inactivation and tumor growth. (B) Tumors inhibit NK cells, but six different ways to reactivate them during tumorigenesis have been uncovered: the use of modified NK cells (chimeric antigen receptor [CAR]-NK), stimulation by IL-15, targeting transforming growth factor β (TGF-β), using antibodies binding the tumor cells and NK cells (antibody-dependent cellular cytotoxicity [ADCC] and bispecific killer engagers [BiKE]/trispecific killer engagers [TriKE]), and inhibition of NKG2A or CD96 to suppress the immunosuppressive NK population.

Adoptive transfer of NK cells as immunotherapy requires their ex vivo expansion and subsequent strong in vivo activity, persistence, and cytotoxicity. NK cells can be derived from stem cells, from peripheral blood NK cells of a healthy donor (allogeneic condition), from the patient (autologous condition), or from the NK-92 cell line. Interestingly, injection of allogeneic NK cells from peripheral blood in combination with HCC cryoablation improved global immune function, reduced AFP expression, and was associated with longer progression-free survival (n = 61).[166] In two phase III clinical trials, NK-based CIK therapies were well tolerated and improved overall survival (n = 200; n = 270).[167,168] Recently, a phase I study reported that administration of allogeneic CIK prevents HCC recurrence after liver transplantation (n = 18, NCT01147380).[169] Similarly, the human NK-92 cell line, which presents high cytotoxic activity and can be expanded under good manufacturing, has shown promising antitumor responses when injected into patients.[170] Two injections of NK-92 cells were sufficient to trigger antitumor responses in patients with treatment-resistant lung cancer (n = 13), and no long-term side effects were observed, indicating that they can be used as a promising therapy.[171] Also, allogeneic NK cells genetically modified to express the chimeric receptor NKG2D-CD3ζ-DAP10 (which includes the receptor, the signaling domain, and the signaling adaptor, respectively) were shown to enhance anti-HCC cytotoxicity in vitro and in immunodeficient tumor-bearing mice.[172]

These last few years, the generation of chimeric antigen receptor (CAR)-T cells has revolutionized cancer treatment. Briefly, the CAR is composed of an artificially modified fusion protein combining an extracellular antigen recognition domain followed by a spacer and transmembrane region and fused to a wide range of intracellular signaling domains. CAR-NK cells are now used to improve the targetability and efficacy of NK cells. In HCC, CAR-modified NK-92 cells were used to specifically target Glypican-3 (GPC3)-positive tumor cells.[173] Recently, NKG2D-CAR-NK cells were able to delay colorectal cancer's progression in three patients with chemotherapy-refractory metastatic colorectal cancer, suggesting the possibility of treating patients with CAR-NK cells[174] (Figure 6B).

The functional rescue of NK cells in HCC could represent another strategy for new immunotherapeutic treatments.[175] Metabolic defects and functional impairment of circulating NK cells in patients with

HCC can be partially attributed to TGF-β signaling.[176] Incubation of NK cells from healthy donors with an anti-TGF-β agent partially restored functional defects of the NK cells, suggesting that TGF-β might represent a suitable target for immunotherapy.[176] Despite its potential toxicity, IL-15 has also been proposed as a potential pharmacological candidate for cancer therapy because of its role in NK cell proliferation, survival, and cytotoxicity[177] (Figure 6B). Alternatively, enhancing the NK cell–mediated antibody-dependent cellular cytotoxicity (ADCC) during tumorigenesis may represent a way to activate NK cells and kill tumor cells.[178–181] There are currently great expectations for bispecific killer cell engagers, which are composed of two antibody fragments, one recognizing a tumor antigen and the other one recognizing CD16a on NK cells to bring them closer and form an immunological synapse to induce the NK cytolytic function.[182]

The discovery of new NK populations allows us to speculate that specific suppression of the immune-modulator NK subset might represent a therapeutic potential. Cultured $CD96^+$NK cells presented low expression of perforin and granzyme B associated with the expression of PD-1, NKG2A, IL-10, and TGF-β. Nevertheless, reversing NK cell exhaustion in vitro by blocking the interaction between CD96 and its ligand CD155 restored the cytotoxicity of NK cells.[183] Cotargeting CD96 with other immunosuppressive receptors may provide a more powerful boost in antitumor immune responses. Recently, monalizumab, a humanized anti-NKG2A antibody, has shown promising results in squamous cell carcinoma of the head and neck by enhancing tumor immunity promoted by both NK and CD8+ T cells.[184] Finally, regorafenib, in addition to its other antitumoral roles, also inhibits the expression of ADAM9 and ADAM10 in HCC cell lines, resulting in higher MICA (NKG2D-ligand) expression at the membrane and lower secreted MICA levels, leading to higher cytotoxic NK cell activity.[185] The combination of regorafenib and an NK cell therapeutic strategy could potentially enhance the antitumor effect of regorafenib.

Harnessing the NK antitumor activity as a novel immunotherapeutic approach has revealed promising effects in solid cancers and hematological malignancies.[163,165,178,186] All these NK-targeting strategies used alone or in combination with other treatments are expected to increase the number and activation of NK cells at the tumor site.

T CELLS LEAD THE ANTITUMOR CHARGE

In liver cancers, $CD8^+$ and $CD4^+$ T cells are enriched within the tumor and in the peritumoral area, respectively.[187] Low intratumoral Treg and high number of activated $CD8^+$ T cells is associated with favorable prognosis in patients (n = 302).[188] Treg cells mediate T cell dysfunction in HCC and their presence within the tumor is associated with worse outcomes.[189] Recently, T cell diversity was dissected by scRNAseq in six patients with HBV^+ treatment-naive HCC.[190] Exhausted $CD8^+$ T cells ($PDCD1^+CTLA4^+$) and Tregs ($TIGIT^+CTLA4^+$) were enriched within the tumor and expressed the LAYLIN (*LAYN*) suppressive marker. Interestingly, T cell receptor (TCR) sequencing revealed that 10% of $CD8^+$ T cells harbored clonal TCRs in blood and normal liver tissues, whereas they reached 30% in tumors. Based on trajectory analyses, exhausted $CD4^+$ and $CD8^+$ T cells were closely linked to intermediate populations expressing granzyme A (GZMA) and K (GZMK) markers, respectively, rather than to effector populations, suggesting potential therapeutic strategies that favor activation instead of exhaustion.[190] Different $CD4^+$ T cell populations, including Th1, Th2, Th17, and Tregs, can be induced in response to a specific balance of cytokines and chemokines, including TGF-β and IL-6.[191]

In HCC, the expression of PD-1 and its ligands PD-L1/2 have been intensively studied. In physiological conditions, their expression is a defense mechanism to prevent the activation of autoreactive T cells and the death of healthy cells.[192,193] Pioneering studies in HCC have shown that PD-1 expression is increased on $CD8^+$ T effector cells and PD-1 interacts with tumors expressing PD-L1/2, which blocks signaling, proliferation, and cytokine secretion of T cells.[194,195] In a cohort of patients infected with HBV, the frequency of circulating $PD\text{-}1^+CD8^+$ T cells increased with disease progression.[194] $CD8^+$ T cells induce PD-L1 expression on hepatoma cells in an IFNγ–dependent manner, which in turn promotes apoptosis of T cells. HCCs with a discrete population of PD-1–high cells are more aggressive but are predicted to respond to anti-PD-1 therapy[195] (Figure 7A). In 2018, James P. Allison and Tasuku Honjo were awarded the Nobel Prize in Physiology or Medicine for the discovery of immune checkpoints, which are targets for cancer immunotherapy.[196] Antibodies against immune checkpoints such as PD-1 and CTLA4 disrupt coinhibitory T cell signaling, thus reactivating the immune response against tumor cells. ICIs against PD-1 (nivolumab and pembrolizumab) and PD-L1 (atezolizumab, durvalumab, and avelumab) have been tested in a series of clinical trials including patients with HCC. A recent meta-analysis of eight trials has revealed that ICIs are more efficient in patients with viral hepatitis compared with non-viral-related HCC, whereas no differences were seen regarding etiology in patients treated with TKI/anti-VEGF.[197] In addition, two NASH-driven HCC cohorts treated with anti-PD-1 or anti-PD-L1 showed reduced overall survival compared with patients with other etiologies,

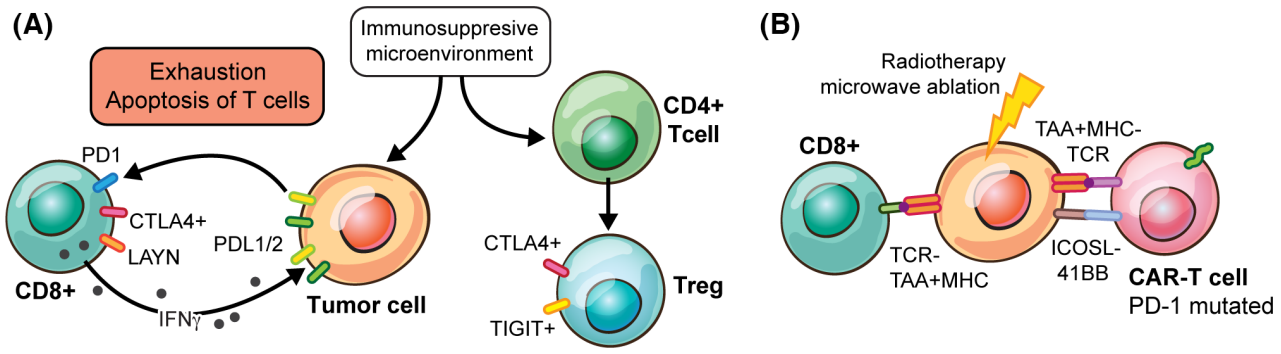

FIGURE 7 T cells lead the antitumor charge. (A) The immunosuppressive environment, notably through the secretion of cytokines such as transforming growth factor β (TGF-β), leads to regulatory T cell (Treg) differentiation, associated with the expression of immune-checkpoint ligands by the tumor cell (programmed death-ligand 1/2 [PD-L1/2]), which cause inhibition of $CD8^+$ T cells. (B) Different generations of chimeric antigen receptor (CAR)-T cells have been designed to recognize the tumor cell, kill it, and remain "nonexhausted" (programmed cell death-1 [PD-1] deletion).

reinforcing the hypothesis that NASH-driven HCC seems less responsive to immunotherapy. A prospective validation is still needed to strengthen these data, given the relatively small number of patients with NAFLD in the current study.[198] Interestingly, in concordance with this work, Dudek et al. showed an increase of unconventional resident activated and exhausted hepatic $CXCR6^+$ $PD\text{-}1^+CD8^+$ T cells in the choline-deficient high-fat diet mouse model and human NASH tissue: these cells participate in tissue autoaggression and may favor HCC development by reinforcing CLD.[198,199]

The majority of HCCs are not enriched in inflammatory TME responses, thus constituting a large group of tumors with potentially little response to ICI.[200] Immune-active HCCs are characterized by active helper $CD4^+$ T and cytotoxic $CD8^+$ T cell infiltration and are prone to respond to ICIs. Conversely, immune-exhausted HCCs are associated with $CD8^+$ T cell exhaustion, an increase of Treg cells, and downregulation of NK cell activators. This group overlaps with the proliferative progenitor subclass and the *CTNNB1*-mutated subclasses G5–G6.[200,201] Our laboratory has reported that *CTNNB1*-mutated tumors undergo immune escape and resistance to ICIs through a decrease in CCL5 cytokine, which impairs DC recruitment and leads to ineffective $CD8^+$ T cell tumor clearance.[202] Finally, radiotherapy enhances tumor immunogenicity, and its combination with ICIs is being evaluated as an approach for HCC (NCT03203304, NCT04611165, NCT03482102).[203] Taken together, it is clear that only a subset of patients can benefit from immunotherapy, necessitating the identification of disease-progression markers associated with robust predictors of ICI response and combination strategies that convert resistant tumors to responsive ones.

$CD8^+$ T cell responses against specific TAAs are considered to be potential immunological antitumor forces, but they are not well amplified in HCC, suggesting an inefficient induction and restricted antigen recognition.[201] Indeed, exhausted TAA-specific $CD8^+$ T cells ($PD\text{-}1^+TIM3^+LAG3^+$) have been described in patients with HCC with an upregulation of activated markers despite low levels of granzyme B and effector cytokines.[131] Interestingly, patients treated with radiofrequency ablation present TAA-specific T cell responses associated with a decrease in MDSCs and reduction in HCC recurrence.[204] Among HCCs, AFP is the most studied TAA because CTL epitopes for AFP were identified at early stages of tumorigenesis.[205,206] AFP is transcriptionally reactivated and highly expressed in 75% of patients with HCC,[207] and serum AFP level is used as a biomarker of the disease because it is inversely correlated with survival.[208] AFP peptide-based immunization has shown only modest clinical responses in a small cohort of patients.[209,210] Human T cells transduced with a specific mouse TCR for HLA-A2/AFP complex recognized $HLA\text{-}A2^+AFP^+$ HepG2 cells, leading to cytokine production and tumor death in vitro and in tumor-bearing immunocompromised mice.[211] An ongoing phase I clinical trial (NCT03132792) is examining the safety of adoptive transfer of engineered T cells recognizing the HLA-A*02–restricted AFP158-166 peptide, FMNKFIYEI.[212] Although HLA-A2 is the most common HLA-A allele in Europe and North America, it is not in Asia.[213] Thus, KWV3.1-TCR specific for AFP2–11-HLA-A*24:02-restricted peptide KWVESIFLIF (AFP2–11) has been designed and was able to kill $AFP^+HLA\text{-}A*24{:}02^+$ tumor cell lines.[214] Recently, microwave ablation in patients with HCC (n = 23) revealed de novo or enhanced tumor-specific immune responses in 30% of patients through enhancing TAA.[215] This response was correlated with long-term survival, supporting the combination of local ablation and immunotherapy. In addition, HCC-TAAs such as GPC3 and AFP are being tested as targets of CAR-T. Infusion of autologous HBV-specific CAR-T cells was able to target HBsAg-expressing HCC cells in a subject with end-stage HCC without exacerbation of liver inflammation or toxicity.[216] GPC3-CAR-T cells were able to eliminate $GPC3^+$HCC cells and tumors in

a patient-derived xenograft[217] and have been recently tested in phase I clinical trial to prove safety and potential efficacy in patients with advanced GPC3$^+$HCC (NCT02395250 and NCT03146234, n = 13).[218] A second and third generation of these CAR-T cells have been generated with the disruption of PD-1 via CRISPR/Cas9 or with the coexpression of the costimulatory molecule ICOSL-41BB[219,220] (Figure 7B). Both improve the persistence and infiltration of CAR-T cells in mice bearing tumors. Additionally, dual-targeted CAR-T cells directed against GPC3 and asialoglycoprotein receptor exerted superior anticancer activity and persistence compared with GPC3-CAR-T cells alone in two GPC3$^+$ASGR1$^+$HCC xenograft models.[221] NKG2D-based CAR-T cells have also been generated and have shown an efficient destruction of NKG2DL$^+$HCC cells in vitro and in NKG2DL$^+$HCC xenografts.[222] A phase II clinical trial (NCT02541370) has recently reported that CAR-T-133 cells, targeting the cancer stem cell marker CD133, present antitumor activity and a manageable safety profile in patients with advanced HCC (n = 21).[223] Finally, two CD19-CAR-T therapies were approved for the treatment of relapsed and refractory pre-B cell acute lymphoblastic leukemia and diffuse large B cell lymphoma by the FDA as examples of first gene therapies.[224,225]

B CELLS JOIN FORCES TO INHIBIT OR PROMOTE CANCER

B lymphocytes possess different functions in ADCC and antigen presentation, and increasing evidence suggests an additional role in regulating innate and adaptive immunity through the production of cytokines.[226] Although B cells were once thought to be passive players in HCC, the role of tumor-infiltrating B cells (TIBs) remains controversial.

An increase in CD20$^+$ B cells in the tumor margin area has been correlated with favorable prognosis and linked to small tumor size, absence of vascular invasion, and increased density of CD8$^+$ T lymphocytes, especially in HBV-associated HCC (n = 120).[227] Along the same line, high densities of B cell subsets prolonged survival in two independent HCC cohorts (n = 619).[228] Plasma cells were the most abundant group, suggesting that B cell responses occur in the TME. Also, plasma cells defined as CD20$^-$CD79α^+ cells were significantly associated with better prognosis.[229,230] Moreover, the density of TIBs was positively correlated with the number and activation status of both T and NK cells and coincided with reduced tumor cell viability (n = 112).[231] Indeed, the density of TIBs correlated positively with the density of apoptotic tumor cells and negatively with tumor cell proliferation. Also, an unsupervised gene expression analysis of full cancer transcriptomes (n = 2158 patients) revealed that the infiltration of CD20 cells and CD79a cells was associated with prolonged survival of patients and secretion of immunoglobulins.[232] Deeper investigations based on immunohistochemistry have highlighted that atypical CD20$^+$ memory B cells (IgD$^-$IgG$^+$CD27$^-$CD38$^-$) possess tumor-killing potential and cooperate with CD8$^+$ T cells, which are responsible for a favorable prognosis.[227] These findings suggest that B cell subsets may enhance the antitumor effect in HCC. Using diethylnitrosamine-induced liver cancer mouse models, it has been shown that T cells prevent initial tumor formation whereas B cells limit growth.[225] Igh6$^{-/-}$ mice lacking B cells develop more and larger tumors than WT animals. B cell depletion with CD20 antibody led to enhanced tumor growth of transplanted hepatoma cells (Hepa1-6) in mice, mainly because of a reduction of CD4$^+$ T cell activation. Moreover, CD20 depletion was also associated with enhanced PD-1 expression on CD8+ T cells, indicating that CD8$^+$ T cell activation can be influenced by CD20$^+$ B cells[225] (Figure 8A).

B cells may also exert a protumorigenic role. A recent study has reported that high infiltration of CD20$^+$ B cells within the tumor correlates with poor differentiation and lower disease-free survival in patients with HCV-induced HCC.[233] B cells secreted proinflammatory cytokines such as TNF-α in the liver of an HCC mouse model (*Mdr2*$^{-/-}$ mice). Thus, in a mouse model of chronic inflammation–driven HCC, IgA$^+$ B lymphocytes supported tumor growth by actively counteracting CD8$^+$ (PD-1$^+$Tim3$^+$) T cell function.[234] PD-L1 blockade was sufficient to decrease liver IgA$^+$IL-10$^+$ cell abundance and increase CD8$^+$ T cells.[234] Other B lymphocytes are implicated in the progression of HCC, including B regulatory cells (Bregs), whose role is being intensely studied regarding their strong immunosuppressive function via the production of IL-10.[235] CD4$^+$ T cell–mediated cytotoxicity was negatively controlled by Tim-1$^+$ IL-10–expressing B cells in a small cohort of HBV-related HCC (n = 28).[236] The Breg population is characterized in humans as CD19$^+$CD24^{hi}CD38^{hi} and in mice as CD19$^+$CD21^{hi}CD23^{hi}IgM^{hi}CD24^{hi}.[237] Overall, Bregs are enriched in the HCC TME and have been associated with tumor progression.[238] Circulating Bregs were accumulated in patients with HCC (n = 80), whereas B memory cells (CD24$^+$CD38$^-$/CD19$^+$) were decreased compared with healthy individuals. In severe combined immunodeficient xenografted mice, displaying a lack of B and T cells, injection of Bregs influenced HCC tumor growth by migrating into the tumor and producing IL-10. Administration of anti-CD154 (CD40L) antibody abolished tumor growth. Inhibition of CD40/CD40L axis in the coculture of Bregs and HCC cells led to a decrease in TGF-β1 and IL-10 but an increase in TNF-α secretion, showing that Bregs promote a strong anti-inflammatory environment.[238] Another Breg subset has been characterized in human HCC by the expression of a high level of PD-1 and IL-10 (CD5^{hi}CD24$^{-/+}$CD27$^{hi/+}$CD38^{dim}).[239]

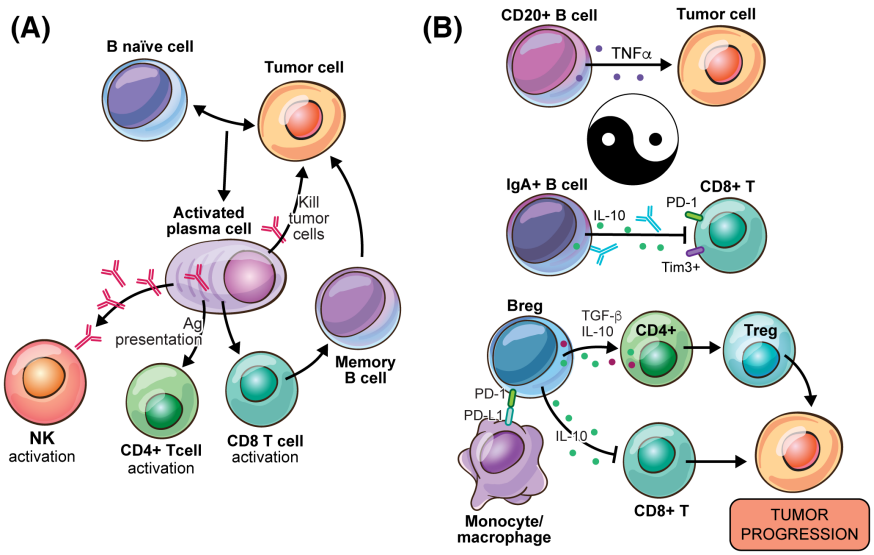

FIGURE 8 B cells join forces to promote or inhibit cancer. (A) Naive B cell interaction with tumor cells triggers B cell differentiation into an activated plasma cell able to kill the tumor cell via the secretion of specific antibodies. Thus, it promotes natural killer (NK) cell activation though the antibody-dependent cellular cytotoxicity (ADCC) mechanism as well as T cell activation. (B) $CD20^+$ B cells can limit growth of established tumors by secreting TNF-α. However, IgA^+ B cells and regulatory B cells (Bregs) inhibit T cells by secreting IL-10 and transforming growth factor β (TGF-β). This immunosuppressive environment also triggers polarization of monocytes and macrophages to reinforce it.

Patients with higher proportions of PD-1^{high} B cells showed early recurrence after 12 months. This study suggests that PD-1^{high} B cells may be activated by PD-$L1^+$ monocytes/macrophages, allowing the production of IL-10 and the subsequent $CD8^+$ T cell inactivation. In another human HCC cohort ($n = 35$), 50% of B cells exhibited $CD20^+FcγRII(CD32)^{low/-}$ activated phenotype, and their accumulation into the tumor positively correlated with patient tumor-node-metastasis stages.[240] Interestingly, activation of this particular subset of B cells was triggered in a CD95L-dependent manner, coordinated by semimature DCs. However, the $CD20^+FcγRII^{high}$ subset is the major source of IL-10 production in mice.[241] Indeed, targeting the CD95 axis could attenuate Breg activation and IL-10 production. A clinical trial based on sorafenib in advanced HCC (n = 62) demonstrated a better prognosis in patients with a reduced Breg ratio in the peripheral blood.[242] These data suggest that the proportion of Bregs in peripheral blood may be indicative of sorafenib efficacy (Figure 8B).

B cell subsets are highly heterogeneous, and some of them coexist within the TME. Further investigation on the B cell repertoire is needed to identify potential targetable subsets and molecules and to explore therapies in future clinical trials.

CONCLUSIONS

HCC tumors present a complex environment with interactions between tumor cells and other cell populations.[243] The TME is composed of a range of immune cells, CAFs, and endothelial cells, fueling cancer cells with growth factors, and facilitating proliferation, immune evasion, and angiogenesis. scRNAseq studies have recently highlighted that the liver TME exhibits a more uniform pattern between patients than HCC tumor cells, suggesting that treating the TME may be a better strategy than treating tumor cells alone to bypass the variability and diversity of tumors.[48,49] This notion is further supported by the fact that the main dominant mutational drivers in HCC remain undruggable.[15] Understanding the role of the TME is gaining increased attention because it plays a significant role in clinical outcomes and response to therapy. The emergence of novel immunotherapies has begun to change the landscape of liver cancer treatment. Last year, the combination of atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF) was FDA approved as a first-line treatment for advanced HCC, representing a new age in HCC treatment.[25] However, only the immune-active subgroup of HCC is believed to respond to therapy, and there is no specific blood marker to easily identify responding patients.[26] In this context, understanding the interactions between oncogenic pathways and immune responses is critical to improving the efficacy of current and future treatments. In addition, understanding the interactions between different immune cells with each other and immune cells with stromal cells, such as HSCs or CAFs, will be critical to therapeutically exploit the TME. For example, it has been shown that depending on the context, senescent HSCs can either restrict or promote tumorigenesis through context-dependent interactions with the immune system.[244,245] In the future, precision medicine will revolutionize HCC therapies, as accumulating evidence suggests that patients

who receive personalized therapy have better clinical outcomes.[246]

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Romain Donne drafted and wrote the article. Amaia Lujambio provided critical feedback and gave approval to the final version.

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CONFLICT OF INTEREST

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